

### **REMARKS**

Claims 1, 14, 27, 40, 71, and 74 have been amended herein. Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76 remain pending. Favorable reconsideration is respectfully requested.

Verbatim support for the recitation of "0.01 to 1°C per second," as added to Claims 1, 14, 27, 40, 71, and 74 is found in the specification at page 16, lines 1-10. No new matter is added.

The following Remarks address the issues presented in the Office Action in order of their appearance.

#### **Rejection of Claims 1-5, 7-18, 20-31, 33-44, 46-52 and 67-76 under 35 USC §112, First Paragraph (Written Description):**

This rejection is respectfully traversed. The Office has taken the position that the term "monolayer" is regarded as new matter because a word search of the specification failed to locate the verbatim terms "monolayer" or "layer."

The rejection is traversed on its face because the Court of Appeals for the Federal Circuit has long made it clear that there is no requirement that a claim recitation have *ipsis verbis* support in the specification to comply with §112, written description. See *Kennecott Corp. v. Kyocera International Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987). See also MPEP 2163.07(a):

By disclosing in a patent application a device that inherently performs a function or has a property, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter. *In re Reynolds*, 443 F.2d 384, 170 USPQ 94 (CCPA 1971); *In re Smythe*, 480 F. 2d 1376, 178 USPQ 279 (CCPA 1973).<sup>1</sup>

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<sup>1</sup> See also *Schering Corp. v. Amgen Inc.*, 222 F.3d 1347, 1352, 55 USPQ2d 1650, 1653 (Fed. Cir. 2000) ("The fundamental inquiry is whether the material added by amendment was inherently contained in the original application."); *TurboCare Div. of Demag Delaval Turbomachinery Corp. v. Gen. Elec. Co.*, 60 USPQ2d 1017, 1023 (Fed. Cir. 2001) ("In order for a disclosure to be inherent, 'the missing descriptive matter must necessarily be present in the application's specification such that one skilled in the art would recognize such a disclosure.'") (quoting *Tronzo v. Biomet, Inc.*, 156 F.3d 1154, 1159, 47 USPQ2d 1829, 1834 (Fed. Cir. 1998)).

In line with the preceding cases, the Office's insistence that the term "monolayer" is new matter for the sole reason that it does not appear verbatim in the specification is, *prima facie*, improper.

Applicants take note, however, that the Office has indicated that "in the absence of any identified support for the term," the claims remain rejected as containing new matter. See page 3, lines 8 and 9, of the Office Action. Applicants therefore take the opportunity to provide the requested support.

Specifically, at page 11 of the specification, it is noted that one strand of the PCR product is bound to the solid surface. At page 11, lines 9 and 10, the specification states "The current binding surface format used is a 96 well microtitre plate that has been coated with streptavidin (available from various manufacturers)." Emphasis added. These commercially-available, streptavidin-coated surfaces comprise monolayers of streptavidin adhered to the surface. As evidence, please see Exhibits 1-8, attached hereto and incorporated herein.

Exhibit 1 is an excerpt from the website of Dynal Biotech (Oslo, Norway and Brown Deer, Wisconsin). Dynal is the maker of DYNABEAD®-brand separation matrices. Exhibit 1 describes Dynal's streptavidin-coated products. The excerpt explicitly states "Analysis and close calculations show that the bead-coating consists of a monolayer of covalently coupled streptavidin." In short, the Dynal product is unquestionably a surface modified to contain a monolayer of streptavidin.

Dynal Biotech is one of many commercial suppliers of such surfaces. For example, Promega Corporation (Madison, Wisconsin) markets a 96-well microtitre plate of the type referenced at page 11 of the present specification. Not coincidentally, Promega's product is marketed under the trademark "SAM<sup>2</sup>". See Exhibit 2, attached hereto and incorporated herein. Exhibit 2 is the product insert literature for Promega's SAM<sup>2</sup>-brand biotin capture membrane. Of particular note is that the very name of the product, SAM, is a well-known acronym for "self-assembled monolayer." (As evidence of the well-known nature of the SAM acronym, see Exhibit 3, which is an excerpt from the [www.acronymfinder.com](http://www.acronymfinder.com) website, a web-based resource that is widely cited by the trademark side of the Patent & Trademark Office.) In short, Promega's

SAM<sup>2</sup>-branded product is a surface modified to contain a self-assembled monolayer of streptavidin molecules.

A slew of other companies make essentially equivalent streptavidin-coated surfaces wherein the streptavidin is in the form of a monolayer on the surface. Examples include Perkin Elmer (see Exhibit 4), Nunc (Exhibit 5), Upstate (Exhibit 6), and Roche Applied Science (Exhibit 7). All of these products are commercially available and readily located by a person of ordinary skill in the art. They are all surfaces modified to contain an immobilized streptavidin monolayer.

Most notably, the specification, at page 24, line 12, indicates that a streptavidin-coated plate purchased from Boehringer-Mannheim was used. In a corporate merger/restructuring, Boehringer-Mannheim was renamed Roche Molecular Biochemicals on March 5, 1998 (see Exhibit 8), which company was then subsequently re-named Roche Applied Science. The product formerly marketed under the Boehringer-Mannheim name is now sold by Roche Applied Science under the trademark StreptaWell (see Exhibit 7).

The significance of the Exhibits 1-7 is that a biotin-labeled DNA, when contacted with a monolayer of immobilized streptavidin molecules, will yield a corresponding monolayer of DNA molecules. This outcome was clearly articulated in the Strohner Declaration, submitted earlier.

On this point, Applicants respectfully submit that the Strohner Declaration has not been accorded appropriate weight. The Office has dismissed the Strohner Declaration on the grounds that the claims do not recite a monolayer of streptavidin. This is irrelevant because it ignores the central fact proven by the Declaration. Namely, the specification clearly describes attaching DNA molecules to the individual wells of a commercially-available, streptavidin-coated, thin-wall microtiter plate. See page 24, lines 10-13 of the specification. The declaration of Strohner confirms that the inevitable result of this attachment is a DNA monolayer as recited in the claims. The use of this term in the claims is therefore not added matter. The evidence presented in the Strohner Declaration, evidence that has been improperly discounted, is that a streptavidin-coated surface does not have any significant third-dimensional structure and DNA attached to a streptavidin monolayer will invariably form a corresponding DNA monolayer.

Regarding the Office's citation of the Jordan et al. reference, Applicants respectfully submit that the Office has quite seriously misinterpreted the disclosure of this reference.

The Office has cited Jordan et al. as evidence that the attachment of DNA to a surface as described in the present specification need not result in a monolayer. However, there are major defects in this reasoning.

*1) The Jordan et al. molecular arrangement is wholly different from that described in the present application:*

The arrangement used in Jordan et al. is completely different from that described in the specification. This is unambiguously confirmed on page 4940, left-hand column of Jordan et al., which states:

This absorption scheme is the reverse of the chemistry used in research reported previously which employs the binding between streptavidin and biotinylated oligonucleotides to immobilise DNA on strepavidin-coated surfaces....

*2) The Jordan et al chemistry is different from that described in the present application:*

Jordan et al. describe the use of thiol chemistry to fix the probe DNA to the surface. Page 4940, right-hand column states: "A thiol coupling surface chemistry that utilizes the self-assembly of alkanethiol monolayers... was employed to immobilize single-stranded DNA at gold surfaces."

In short, there simply is no teaching in Jordan et al. of single-stranded DNA being directly immobilized on a surface using streptavidin. Rather, in the Jordan et al. method, streptavidin is used to enhance the SPR signal by binding the streptavidin to a biotinylated oligonucleotide which hybridizes to the probe sequence already immobilized on the surface.

Jordan et al therefore provides no support for the assertion that single-stranded DNA bound via streptavidin linkages to a microtire plate as described on page 20 of the specification may form anything other than a monolayer.

3) *The DNA which is attached to the solid surface of Jordan et al. is a monolayer, not a multilayer:*

Contrary to the assertion made by the Office, the Jordan et al. reference proves Applicants' position, rather than refutes it. The Office asserts that Jordan et al. discloses something other than a monolayer. But a close reading of Jordan et al. reveals that Fig. 1 of the reference depicts a sequence of steps taken over time, each step yielding a corresponding monolayer. In short, the "multilayer" assemblies described in Jordan **do not** contain multilayers of the DNA (termed probe DNA or P DNA) which is attached to the glass surface. Rather, and in sharp contrast to the Office's interpretation, the probe DNA is **explicitly** stated in Jordan et al. as forming a "**probe DNA monolayer**." (See Jordan et al., page 4940, right-hand column, middle of first full paragraph. Even where the binding of other molecules (in the second and third steps) leads to the formation of multiple layer assemblies, the base layer of each assembly is a P DNA monolayer, and each subsequent layer is a corresponding monolayer. In short, the various layers depicted in Fig. 1 of Jordan et al. are simply a series of monolayers, one on top of the other.

4) *The formation of Jordan's multiple layer construct requires special techniques and reagents:*

In the initial experiments of Jordan et al, as exemplified in Fig 1, a "BC DNA" layer and a streptavidin layer are added to the initial "P DNA" monolayer. The resulting structure is a series of corresponding (albeit discontinuous) monolayers. In short, to enhance the SPR signal, the authors of Jordan et al. deliberately produce "multilayer" assemblies using special techniques and reagents. But, as clearly shown in Fig. 1, all of the "BC DNA" is situated within a monolayer unto itself (as is the "P DNA" below it, and the "SA" layer above it).

The streptavidin/DNA layers are built up by sequentially adsorbing two strands of complementary, biotinylated linker oligonucleotides (BL3 and BC3 of Table 1) (see page 4945, right-hand column). No such linker oligonucleotides are used in the methods described in the present specification.

Further still, the DNA /DNA multilayers of Jordan et al. are assembled using two specific oligonucleotides (C4a and C4b; Figure 8) in which the 5' half of C4a oligo is complementary to the 3' half of C4b oligo (and the 3' half of C4a oligo is complementary to the other half of the C4b oligo). Oligonucleotides with these specialized sequence arrangements are not used in the methods described in the present specification.

The methods described on page 24 of the specification employ only a biotinylated PCR product and a monolayer of streptavidin coated to a multi-well plate. No special reagents are employed or described that would yield multilayer assemblies like those described in Jordan et al. The inherent result of the methods described in the specification, as evidenced by the Strohner Declaration and the Exhibits attached hereto is a monolayer of single-stranded DNA molecules adhered to a monolayer of streptavidin adhered to a surface.

In summary, it is therefore simply incorrect on the basis of Jordan et al. to state that PCR products bound to the solid surface of a microtiter plate as described in the present specification *might* form multilayer structures. They won't (as shown by the Strohner Declaration and the attached Exhibits). Such structures are only formed in the presence of specifically designed reagents, such as those described in Jordan et al. The inherent result of the methods described in the specification is a monolayer of single stranded DNA molecules.

Applicants therefore respectfully submit that the rejection of Claims 1-5, 7-18, 20-31, 33-44, 46-52 and 67-76 under 35 USC §112, first paragraph is improper. Withdrawal of the same is respectfully requested.

**Priority Claim:**

The parent/priority applications GB9821989.2 and PCT/GB99/03329 describe the same system as disclosed in the present application. Thus, for the reasons noted in the immediately preceding section of this Response, both of these priority documents provide §112(1) support for the term "monolayer." Therefore, the present claims are entitled to priority to GB9821989.2 and PCT/GB99/03329 in accordance with §119 and §365. (See also MPEP 201.11.)

Applicants therefore submit that the denial of priority is improper. Applicants request that the Office acknowledge that the current claims are entitled to priority from both parent applications.

**Rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383 and U.S. Patent 6,174,670 to Wittwer:**

This rejection is traversed on several grounds. The overall gist of the Office's position is that it would be *prima facie* obvious to one of skill in the art to employ the SYBR Green markers of Wittwer in the method of Stimpson, to thereby arrive at the presently claimed invention. This assertion fails on several grounds:

*1) Motivation from Wittwer:*

The Examiner alleges that Wittwer teaches that the SYBR intercalator "is useful in the particular assay employed by Stimpson." This is wholly incorrect and is unsupported by either the Wittwer or Stimpson references. Wittwer employs SYBR Green in the monitoring and quantitation of PCR and has nothing whatsoever to say or suggest regarding solid-phase hybridization.

Specifically, Wittwer states, at column 23, lines 9-14:

SYBR™ Green I is a preferred double-strand-specific dye for fluorescence monitoring of PCR, primarily because of superior sensitivity, arising from greater discrimination between double-stranded and single-stranded nucleic acid. SYBR™ Green I can be used in any amplification and is inexpensive. In addition, product specificity can be obtained by analysis of melting curves, as will be described momentarily.

The teaching of "superior sensitivity" is alleged to provide motivation to use SYBR Green in the Stimpson method. However, this comment is taken wholly out of the context of the Wittwer patent. SYBR Green is preferred in Wittwer "for fluorescence monitoring of PCR" and Example

2 of Wittwer (starting at column 22, line 28) shows that SYBR Green is more sensitive than ethidium bromide and acridine orange for this purpose, but not in any other context.

There simply is no teaching in Wittwer that SYBR Green is a "very sensitive detection molecule" or that the alleged "superior sensitivity" of SYBR Green is sufficient to allow its use in other, unrelated applications, such as in monitoring solid-phase hybridization.

Indeed, even though PCR amplification necessarily results in massive amounts of amplified product in solution, the signal produced by SYBR Green is only detectable in Figure 20 of Wittwer after about 20 rounds of exponential amplification. Hence, although its sensitivity is apparently better than ethidium bromide and acridine orange in monitoring PCR reactions, there is nothing to suggest that SYBR Green has the sensitivity required for use in solid-phase hybridization of short oligonucleotides as described in the present specification.

There is no embodiment or suggestion of an embodiment in Wittwer in which SYBR Green is used for allelic discrimination, even in solution. Columns 14 and 46, which are both cited as referring to allelic discrimination, do not use SYBR Green, but rather employ resonance energy transfer probes. Indeed Wittwer specifically states, at column 42, lines 52-59:

When sequence-specific detection and quantification are desired, resonance energy transfer probes can be used instead of double-strand-specific DNA dyes. The  $T_m$  of hybridization probes shifts about 4-8.degree. C. if a single base mismatch is present. If a hybridization probe is placed at a mutation site, single base mutations are detectable as a shift in the probe melting temperature.

In other words, when sequence-specific detection is required, Wittwer explicitly directs that resonance energy transfer probes should be used instead of dyes such as SYBR Green.

The closest that Wittwer comes to sequence-specific detection is the use of melt curves of amplified PCR products in order to distinguish PCR products which have completely unrelated sequences. Intercalating agents such as SYBR Green are known to have a significant effect on the stability of duplex DNA molecules and there is no teaching that subtle differences, such as a single base-pair change in otherwise identical sequences, can be distinguished using SYBR Green (and still less single base-pair differences between very small molecules immobilized on a solid surface).



Thus, the Office's assertion that Wittwer teaches sequence-specific detection using SYBR Green are based not on a "less preferred embodiment" but on an amalgam of different embodiments which have been cobbled together by improperly using the Applicants' own disclosure in a hindsight reconstruction of the prior art. The Office, however, is not free to use Applicants' own disclosure to provide the required motivation or suggestion that is absent from the applied references.

It is therefore respectfully submitted that there is no motivation provided by the Wittwer et al. patent for the skilled person to use SYBR Green in the methods of Stimpson.

*2) Motivation from Stimpson:*

Stimpson et al emphasize that fluorescence-based systems are insensitive and therefore provides an alternative optical wave guide system which improves sensitivity.

Stimpson specifically and purposefully sets out to overcome the problems associated with fluorescence by employing an optical wave guide. There is no broader disclosure or non-preferred embodiment which might teach the skilled person to disregard the entire paper and adopt a completely different approach. This is clearly evidenced by the declaration of Dr Baldeschwieler, who was senior investigator on the Stimpson paper. The very title of the paper clearly sets forth the entire disclosure of the Stimpson et al. paper: "Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides."

A skilled person would receive no motivation whatsoever from Stimpson to attempt to replace the optical wave guide system, which is the central subject of the paper, with a different system, still less a system using SYBL Green as described by Wittwer et al. In short, there is no motivation or suggestion provided by either Wittwer et al. or Stimpson et al. to combine these two references in the first place.

3) *Expectation of Success:*

Even if Wittwer and Stimpson are combined, the **combined** teaching of the two references **does not** provide a skilled person a reasonable expectation of success in using SYBR Green in a solid-phase method of Stimpson.

The combination fails to provide a reasonable expectation of success because Wittwer uses SYBR Green to monitor the amplification **in solution** of nucleic acids of 110 bp or larger. Stimpson concerns the solid-phase hybridization of 15-mer oligonucleotides. A skilled person **is not** taught or given any reasonable expectation of success by the combined teaching of Stimpson and Wittwer whether an intercalating agent, in particular a double-strand specific intercalating agent such as SYBR Green, would bind to a short 15-mer sequence. Moreover, the combined references provide no indication that even if binding did take place, whether the sensitivity provided would be sufficient for detection over the background signals from inter-target interactions. The combined references provide absolutely zero guidance on these matters. The person of ordinary skill in the art would thus be faced with an undue amount of experimentation, with absolutely no likelihood of success.

For allele discrimination, sudden and consistent denaturation is required to produce detectable differences in melting curves between wild-type and mismatch probe/target complexes. The effect of an intercalating agent (as opposed to a label) on the denaturation characteristics of the probe/target complex simply is not predictable to a skilled person prior to the development of present invention. Indeed the stabilizing effect of an intercalating agent on the duplex structure might be expected to mask subtle differences in denaturation characteristics caused by sequence mismatches.

In short, replacing a selenium **label** (as taught by Stimpson et al.) with an **intercalating agent** (as taught by Wittwer) involves a considerable amount of additional experimentation, with no guidance at all from the applied references (taken alone or in combination). A skilled person therefore would have no reasonable expectation of success in the absence of such further work.

The Office asserts that the combination of Stimpson and Wittwer would overcome problems with sensitivity levels in solid-phase fluorescent hybridization systems. However, this

is pure speculation on the part of the Office. The evidence of Wittwer that SYBR Green is slightly better than two other dyes in monitoring PCR in solution in no way provides any reasonable expectation that SYBR Green would overcome the extreme problems of low sensitivity levels in solid-phase fluorescent hybridization systems. This is even more apparent due to the fact that Stimpson et al. uses a selenium label in combination with an optical wave guide to detect the signal that is the basis of the Stimpson et al. protocol. Even if the references themselves supported their being combined (they do not), the combined teaching of the two references does not provide nearly enough guidance for a highly skilled practitioner to conclude that Stimpson's method would work using SYBR Green or any other intercalating dye.

Moreover, even if an improvement in sensitivity was observed in a completely different context (*e.g.*, solution-phase PCR reactions as described by Wittwer et al.), there is nothing to indicate that this improvement would be enough to overcome the sensitivity problems of solid-phase fluorescent hybridization systems. Note that the sensitivity problem is addressed by Stimpson by switching away from fluorescence, and adopting light-scattering using a selenium label. See page 6379 of Stimpson, right-hand column, first paragraph. Therefore, there is simply no technological reason a skilled artisan would combine Stimpson with Wittwer because Stimpson approach explicitly abandons the use of dyes as being insufficiently sensitive to acquire an unambiguous signal quickly.

*4) The combination of Stimpson and Wittwer does not Yield the Claimed Invention:*

Even if a skilled person were to (somehow) use the SYBR Green dye in the melting curve analysis of Stimpson, the combination still does not yield the claimed invention.

The teaching of Wittwer is restricted to molecules in solution. Stimpson describes DNA chips which have 15-mer oligonucleotides attached thereto. In contrast, the present claims require a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface....

The skilled person cannot derive this feature from the teachings of either Stimpson or Wittwer, taken alone or in combination. The combined references are simply silent on this positively recited feature of the claimed invention. Furthermore, the present application teaches that the bound probe arrangement (rather than bound target) is problematic and does not allow allelic discrimination (see page 18, line 25, to page 19, line 27 of the present specification). These problems are resolved by the claimed bound target arrangement.

In conclusion, there is no motivation from either Stimpson or Wittwer to make the alleged combination in the first instance, nor any reasonable expectation of success if the combination is made. Furthermore, the combination does not teach or suggest the invention as positively recited by the claims as amended.

Applicants therefore submit that the rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of Stimpson et al. and Wittwer et al. is improper. Withdrawal of the same is respectfully requested.

**Rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383, U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 6,048,690 to *Heller et al.*:**

This rejection is traversed on many of the same grounds recited in the prior section, all of which is incorporated herein. To recap briefly, the Wittwer et al. patent is limited entirely to detection of PCR amplification products, in solution, using a dye such as SYBR Green. Contrary to the assertion made by the Office, the Wittwer et al. patent does not teach or suggest using this dye to detect sequence-specific differences. Quite the contrary, Wittwer et al. explicitly teaches, at column 42, lines 50-60, that when sequence-specific detection is desired resonance energy transfer probes are to be used "instead of double-strand-specific DNA dyes." There is nothing ambiguous about Wittwer's quoted statement. Wittwer explicitly teaches that for this type of detection, a resonance energy transfer probe is to be used, and not a dye. The Stimpson et al. approach uses a wave-guide and a selenium light-scattering label to detect differences in DNA sequence. By Stimpson's own admission, the selenium dye was selected because "the amount of

fluorescence label on the surface of a [DNA] chip is quite low." Stimpson, page 6379, right-hand column. There is simply no technological reason to combine these two references because Wittwer explicitly teaches that the dyes are not suitable for detection sequence-specific differences, and Stimpson et al. explicitly teaches that the fluorophores are not suitable for their purposes either. Both references explicitly teach that using a dye such as SYBR Green for detecting differences in DNA sequence is not likely to be successful. Therefore, there is no motivation to combine these two references in the first place.

Further combining Wittwer and Stimpson with the Heller document does not cure the shortcomings of the Wittwer/Stimpson combination because Heller also does not address using an intercalator in the context of solid-phase DNA analysis. This shortcoming is shared by all three documents.

The Office cites Heller for its teaching of immobilizing oligonucleotides to arrays using the biotin/streptavidin system. Applicants readily acknowledge that the biotin/streptavidin interaction has been used extensively, in a host of biological assays. The point, however, is that the references to Wittwer and Stimpson do not teach or suggest arriving at a method wherein an intercalating dye is used. Thus, the full combination Stimpson, Witter, and Heller fails to render obvious the invention as claimed.

It is therefore respectfully submitted that the rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 under 35 USC §103(a) in view of Stimpson et al., Wittwer et al., and Heller et al. is improper. Withdrawal of the same is respectfully requested.

**Rejection of Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of *Stimpson et al.* (1995) PNAS 92:6379-6383, U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 5,789,167 to *Konrad et al.***

This rejection is traversed on the same grounds recited in the prior two sections, all of which is incorporated herein. To recap briefly, the Wittwer et al. patent is limited entirely to detection of PCR amplification products, in solution, using a dye such as SYBR Green. Contrary to the assertion made by the Office, the Wittwer et al. patent **does not** teach or suggest using this dye to detect sequence-specific differences. Quite the contrary, Wittwer et al. explicitly teaches, at column 42, lines 50-60, that when sequence-specific detection is desired resonance energy transfer probes are to be used "instead of double-strand-specific DNA dyes." There is nothing ambiguous about Wittwer's quoted statement. Wittwer explicitly teaches that for this type of detection, a resonance energy transfer probe is to be used, and not a dye. The Stimpson et al. approach uses a wave-guide and a selenium light-scattering label to detect differences in DNA sequence. By Stimpson's own admission, the selenium dye was selected because "the amount of fluorescence label on the surface of a [DNA] chip is quite low." Stimpson, page 6379, right-hand column. There is simply no technological reason to combine these two references because Wittwer explicitly teaches that the dyes **are not** suitable for detection sequence-specific differences, and Stimpson et al. explicitly teaches that the fluorophores **are not** suitable for their purposes either. Both references explicitly teach that using a dye such as SYBR Green for detecting differences in DNA sequence **is not** likely to be successful. Therefore, there is no motivation to combine these two references in the first place.

Combining Stimpson and Wittwer with Konrad does not cure the fundamental shortcomings of the combined teaching of Stimpson and Wittwer. In short, the Konrad patent is cited solely for its description of typical DNA hybridization conditions. But **the full combination** of Stimpson, Wittwer, and Konrad fails entirely to teach or suggest that an intercalating dye can be used in a solid-phase format method as presently claimed. This is because Wittwer explicitly teaches that the dyes **are not** suitable for detection sequence-specific differences, and Stimpson et al. explicitly teaches that the fluorophores **are not** suitable for their purposes either. Both of

these two references explicitly teach that using a dye such as SYBR Green for detecting differences in DNA sequence is not likely to be successful. The Konrad patent is silent on the matter. Thus, there is no motivation for combining the three references, and the combination, if made, does not suggest the claimed invention (as argued *supra*).

Applicants therefore submit that the rejection of Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of Stimpson et al., Wittwer et al., and Konrad et al. is improper. Withdrawal of the same is respectfully requested.

**In Closing:**

If any questions regarding the application arise, please contact the undersigned attorney. Telephone calls related to this application are welcomed and encouraged. The Commissioner is authorized to charge any fees or credit any overpayments relating to this application to deposit account number 18-2055.

For the Applicant,



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## A Monolayer of Covalently Coupled Streptavidin

Analysis and close calculations show that the bead-coating consists of a monolayer of covalently coupled streptavidin, presenting a perfect environment for the binding of biotinylated targets. As opposed to multilayers of streptavidin, a monolayer does not introduce the risk of streptavidin being sterically hidden and un-available for binding of your ligand/target. The absence of excess physically adsorbed streptavidin ensures superior reproducibility and low batch-to-batch variations. The monolayer on Dynabeads<sup>®</sup> Streptavidin also secure that only neglectible amount of streptavidin will have the possibility to leak (<0.2% of total streptavidin attached after 2 months at 37°C).

There are three different streptavidin-coated Dynabeads<sup>®</sup> available from Dynal Biotech.

- Dynabeads<sup>®</sup> M-280 Streptavidin (Prod. No 112.05/06 and 602.10) is the product most frequently chosen.
- The more hydrophilic Dynabeads<sup>®</sup> M-270 Streptavidin (Prod. No 353.02/13) is suitable for nucleic acid applications with extreme demands, and has a more negative charged surface at pH =7.
- Dynabeads<sup>®</sup> MyOne™ Streptavidin (Prod.No. 650.01, 650.02 and 650.03) are similar to the Dynabeads<sup>®</sup> M-270 Streptavidin in their degree of hydrophilicity. But in contrast to the two 2.8 µm beads mentioned above, the MyOne™ beads are one micron in diameter. The Dynabeads<sup>®</sup> MyOne™ Streptavidin have a high binding capacity and superior performance in automated systems, and are optimal for IVD assays.

All Dynabeads<sup>®</sup> show excellent results in reproducibility and stability.

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# SAM2® Biotin Capture Membrane



Technical Bulletin No. 547

INSTRUCTIONS FOR USE OF PRODUCTS V2861 AND V7861. PLEASE DISCARD PREVIOUS VERSIONS.

All technical literature is available on the Internet at [www.promega.com](http://www.promega.com)

Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

I. Description .....	1
II. Product Components .....	2
III. SAM2® Biotin Capture Membrane Background and Characteristics .....	3
A. Background .....	3
B. Membrane Characteristics .....	3
IV. Procedure for Use of SAM2® Biotin Capture Membrane in Kinase Assays .....	5
V. Related Products .....	6
VI. References .....	7

## I. Description

The SAM2® Biotin Capture Membrane<sup>(a)</sup> binds biotinylated molecules based on their affinity for streptavidin. The proprietary process by which the SAM2® Membrane is produced results in a high density of streptavidin on the filter, providing rapid, quantitative substrate binding in the nmol/cm<sup>2</sup> range. In addition, the Membrane has been optimized for low nonspecific binding. Figure 1 outlines the procedure for use of the SAM2® Biotin Capture Membrane with biotinylated substrate molecules as used in Promega's SignaTECT® Protein Kinase Assay Systems<sup>(a)</sup>.

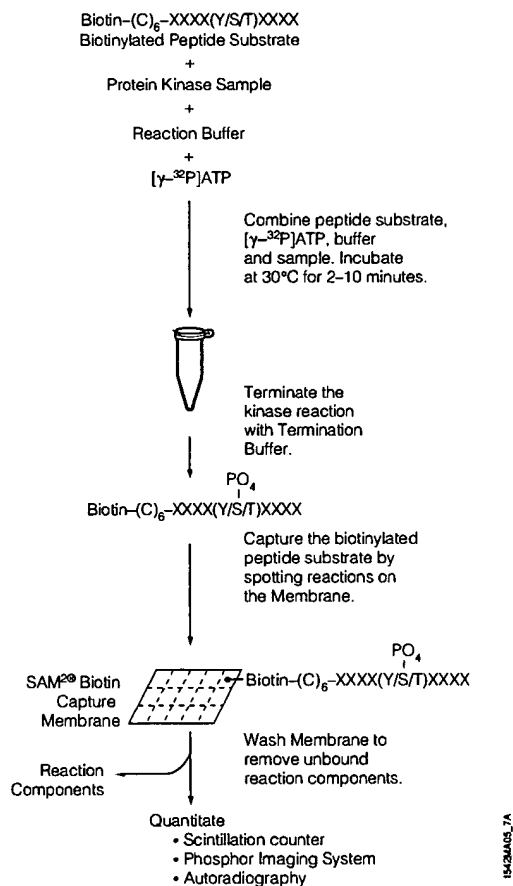
The Membrane is available either as a large, prenumbered, partially cut sheet (approximately 10.5 × 15.0cm; Cat.# V2861) or as a smaller, uncut sheet (approximately 7.6 × 10.9cm; Cat.# V7861). The partially cut Membrane (Cat.# V2861) allows easy separation into 96 individual squares and is designed for small-scale experiments where high binding capacity is required. The uncut sheet (Cat.# V7861) can be analyzed as a whole Membrane or may be cut into the size desired. The uncut Membrane allows for sample application using a multichannel pipettor. Both Membranes may be analyzed using phosphorimaging, autoradiography or scintillation counting to quantitate results. The Membranes have also been used successfully with chemiluminescence detection techniques. The use of fluorescence for detection of captured molecules is not recommended at this time.

The SAM2® Membrane, used as recommended in this technical bulletin, provides a number of advantages over other commercially available streptavidin products. These advantages include:

- **Versatility:** Analysis of biotinylated substrates can be applied to a wide variety of substrate types without the need to optimize each substrate for binding to a matrix. Available in 96-square (partially cut) and solid-sheet (uncut) formats, the user can perform experiments with a wide array of sample numbers and sizes without changing the analysis technique.



- **Specificity:** The combination of protein denaturant and high salt washes minimizes nonspecific binding to the Membrane without interfering with the high affinity interaction between streptavidin and biotin.
- **High Signal-to-Noise Ratios:** The stringent washing conditions employed assist in attaining very low background counts.



**Figure 1. Flow diagram depicting use of the SAM<sup>2</sup>® Biotin Capture Membrane for analysis of kinase activity as used in Promega's SignaTECT® Protein Kinase Assay Systems.**

## II. Product Components

Product	Size	Cat.#
SAM <sup>2</sup> ® Biotin Capture Membrane	96 samples	V2861
SAM <sup>2</sup> ® Biotin Capture Membrane (uncut)	7.6 × 10.9cm	V7861

**Storage Conditions:** Store the SAM<sup>2</sup>® Biotin Capture Membrane in the resealable bag. The Membrane is stable for up to 6 months at -20°C and can be stored at 4°C for up to four weeks.

### **III. SAM<sup>2</sup>® Biotin Capture Membrane Background and Characteristics**

#### **A. Background**

It is frequently desirable in molecular biology and enzymatic analysis to separate a specific substrate from other compounds in a reaction mix. This separation is usually accomplished using a solid matrix that selectively binds the substrate. The matrices, which are often based on ionic or metal ion interactions, are only grossly selective and thus are prone to undesirable variations in performance when different substrates, enzymes or washing conditions are used (1,2). Potential problems include variations in: 1) background and signal intensities depending upon the degree of washing; 2) binding affinity of the substrate based on composition; 3) substrate specificity due to alteration of the substrate to achieve efficient matrix binding. In addition, variations in performance with standard matrices can occur due to the detection of signal from miscellaneous substrates present in complex samples such as crude cell or tissue extracts, which can bind nonspecifically to the matrix (2,3).

Some assays have circumvented these problems by using the high-affinity streptavidin:biotin interaction ( $K_d = 10^{-15}M$ ) to separate the substrate from other reactants (4). This has been accomplished using biotinylated substrates and streptavidin-coated plates or beads. Unfortunately, the limited capacity of streptavidin-coated plates and streptavidin-coated beads places restrictions on the parameters of the assay, thereby limiting the utility of these formats. For example, many enzymes, particularly protein tyrosine kinases, have high  $K_m$  values for peptide substrates, frequently above  $25\mu M$  and as high as  $1mM$  (5). To work at maximal sensitivity (near  $V_{max}$ ) the peptide substrate concentrations must be at least 3 times greater than the  $K_m$ . The binding capacity of commercially available streptavidin-coated plates and beads is generally at least one order of magnitude below this desired capacity. Even if the enzyme activity were sufficiently high to allow suboptimal substrate concentrations to be used at the 96 sample level, further restrictions would be encountered when using miniaturization to 384 samples or higher density arrays.

#### **B. Membrane Characteristics**

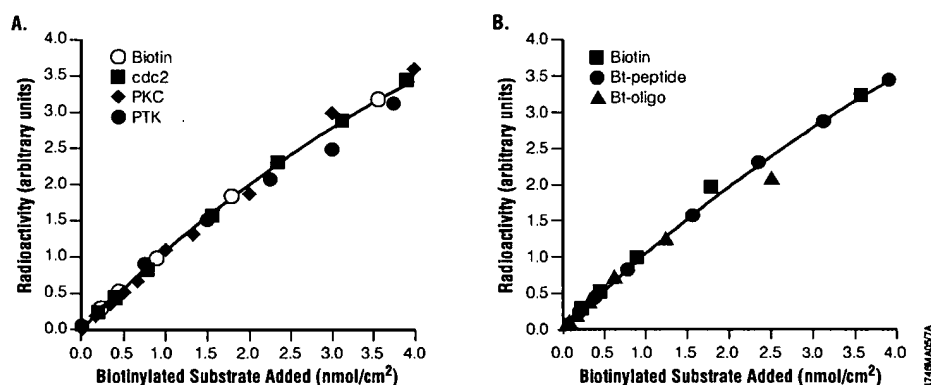
The SAM<sup>2</sup>® Membrane overcomes the problems described above by providing the binding capacity to work at optimal conditions at the 96 sample level while retaining sufficient signal-to-noise ratios to allow miniaturization to higher sample density arrays. The binding of biotin to streptavidin is rapid and strong; binding of the biotinylated molecules to the SAM<sup>2</sup>® Membrane occurs within 30 seconds of sample application. Once formed, this association is unaffected by extremes in pH, temperature, organic solvents, ionic and nonionic detergents and denaturing agents (Table 1; 4).

The 96-square-sheet format (Cat.# V2861) is prenumbered and partially cut so that individual squares can be easily identified, separated and placed into scintillation vials or left intact and quantitated by phosphorimaging or by conventional autoradiography. The uncut sheet (Cat.# V7861) can be utilized for multiple samples in the solid-sheet format, or it can be custom cut to accommodate various sample size and sample number specifications.

**Table 1. Stability Data for the SAM<sup>2</sup>® Biotin Capture Membrane\*.**

Factor	Range Compatible with the SAM <sup>2</sup> ® Membrane
Organic solvents	95% ethanol
Detergents	1% SDS, 1% Chaps, 1% Triton® X-100, 1% Tween® 20, 1% Tween® 40
Denaturing agents	5M guanidine hydrochloride, 2M urea
pH	2.0–10.0
Ionic strength	0–5M NaCl
Binding of streptavidin:biotin	$K_d = 10^{-15}M$
Binding time	<30 seconds
Background counts	0.02–0.1%

\*See reference 6



**Figure 2. Binding of biotin and various biotinylated substrates to the SAM<sup>2</sup>® Biotin Capture Membrane. Panel A:** Binding of several different biotinylated peptide substrates and biotin alone to the SAM<sup>2</sup>® Membrane was compared. The indicated amounts (x-axis) of radioactive biotin and biotinylated peptide substrates for cdc2, PKC and PTK in 2.5M guanidine-HCl, were spotted onto the SAM<sup>2</sup>® Membrane, washed 4X in 2M NaCl, 4X in 2M NaCl plus 1% H<sub>3</sub>PO<sub>4</sub> and 2X in water, were dried and the results quantitated by scintillation counting. **Panel B:** Radioactive biotin and biotinylated peptide (Bt-peptide) were spotted onto the Membrane and washed as stated above. Radioactive, biotinylated oligonucleotide (Bt-oligo) in water was spotted onto the SAM<sup>2</sup>® Membrane, washed 4X in 1% SDS, 2X in water, 4X in 2M NaCl and 2X in water, dried and the results quantitated by scintillation counting.

Studies performed in our laboratories have tested the SAM<sup>2</sup>® Membrane with many different biotinylated peptides and oligonucleotides. We have shown that the binding affinity of the Membrane for these substrates is similar to the affinity of the Membrane for biotin alone (1–3). In addition, binding of biotinylated molecules to the SAM<sup>2</sup>® Membrane occurs independently of amino acid or molecular sequence (Figure 2). This property allows the comparison of multiple peptide substrates, which is especially important for those peptide substrates that do not bind well to standard matrices (7–9).

#### IV. Procedure for Use of SAM<sup>2</sup>® Biotin Capture Membrane in Kinase Assays

The following procedure is recommended for use of the SAM<sup>2</sup>® Membrane in kinase assays with biotinylated peptide substrates. Please note that you will need to optimize the buffers and washing protocol for use of the Membrane with other types of molecules.

1. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the partially cut SAM<sup>2</sup>® Membrane. Alternatively, the squares may remain connected as a sheet to minimize handling. When working with the uncut Membrane (Cat.# V7861), either cut into individual pieces or handle as a whole sheet. Return any unused Membrane to the resealable bag at 4°C or –20°C.
2. After completion of the protein kinase reactions, terminate with 0.5 volume of 7.5M guanidine hydrochloride solution in water (final concentration of 2.5M guanidine hydrochloride).
3. When using the partially cut Membrane (Cat.# V2861), apply 0.1–25µl of the terminated kinase reaction (≤2nmol of peptide) to an individual Membrane square or apply a maximum of 15µl per square if the squares are still connected. For the uncut Membrane (Cat.# V7861), apply a substrate concentration of ≤1.3nmol/cm<sup>2</sup> (see Note below). If applying samples with a multichannel pipettor, the maximum volume applied should be ≤5µl. Allow the samples to absorb to the Membrane; there is no need to dry the Membrane completely before washing.

**Note:** It is possible to bind more than 1.3nmol/cm<sup>2</sup> and retain a linear binding response. The linear binding response above 1.3nmol/cm<sup>2</sup> will depend upon the assay being performed and must be determined by the user (Figure 2).

4. Place the SAM<sup>2</sup>® Membrane squares or the intact sheet containing samples into a washing container. Wash, using a minimum of 100ml of each solution, changing solutions after each wash. Using an orbital platform shaker set on low speed or by manual shaking, follow this washing procedure:

Wash 1 time for 30 seconds with 2M NaCl.  
↓  
Wash 3 times for 2 minutes each with 2M NaCl.  
↓  
Wash 4 times for 2 minutes each with 2M NaCl in 1% H<sub>3</sub>PO<sub>4</sub>.  
↓  
Wash 2 times for 30 seconds each with deionized water.

**Total wash time <20 minutes.**

 **Do not**  
exceed 30µl per square.

**Notes:**

If using radioisotopes, dispose of the radioactive wash solution in accordance with the regulations of your institution.

More or less washing may be appropriate to achieve acceptably low background counts; this should be determined empirically.

For rapid drying, a final 15-second, 95% ethanol wash can be used. Longer washes with ethanol may cause the ink to run slightly.

5. Dry the SAM<sup>2</sup>® Membrane squares on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature 30–60 minutes. (If the SAM<sup>2</sup>® Membrane has been washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)
6. **Analysis by Scintillation Counting:** If you are using radioisotopes and the SAM<sup>2</sup>® Membrane (Cat.# V2861) is still intact, separate the squares using forceps, scissors or a razor blade and place into individual scintillation vials. Add scintillation fluid to the vials and count. The uncut Membrane (Cat.# V7861) may be cut into sample pieces and each piece analyzed in individual vials after addition of scintillation cocktail.

**Analysis by Phosphorimaging:** Alternatively, the SAM<sup>2</sup>® Membrane may remain intact and the intact SAM<sup>2</sup>® Membrane may be analyzed using a phosphorimaging system.

**V. Related Products**

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
SAM <sup>2</sup> ® 96 Biotin Capture Plate <sup>(a)</sup>	96 well plate	V7541
	5 × 96 well plates	V7542
SignaTECT® Protein Tyrosine Kinase Assay System <sup>(a)</sup>	96 reactions	V6480
SignaTECT® Protein Kinase C (PKC) Assay System <sup>(a)</sup>	96 reactions	V7470
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System <sup>(a)</sup>	96 reactions	V7480
SignaTECT® DNA-Dependent Protein Kinase Assay System <sup>(a)</sup>	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaMKII) Assay System <sup>(a)</sup>	96 reactions	V8161
SignaTECT® cdc2 Protein Kinase Assay System <sup>(a)</sup>	96 reactions	V6430
PepTag® Non-Radioactive PKC Assay <sup>(b)</sup>	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay <sup>(b)</sup>	120 reactions	V5340

## VI. References

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<sup>(a)</sup>U.S. Pat. No. 6,066,462 has been issued to Promega Corporation for quantitation of protein kinase activity.

<sup>(b)</sup>U.S. Pat. No. 5,580,747 has been issued to Promega Corporation for a non-radioactive enzyme assay.

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All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.



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## Acronyms &amp; Abbreviations (most common first)

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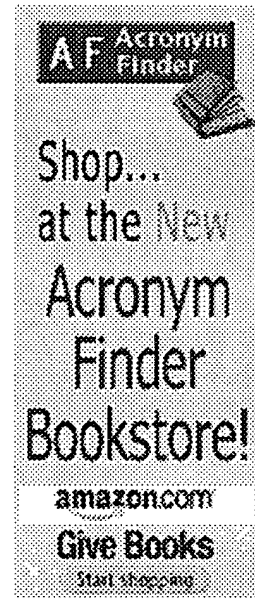
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## Acronym Definition

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<b>SAM</b>	Secure Access Module	
<b>SAM</b>	Security Account Manager	
<b>SAM</b>	Security Authentication Module	
<b>SAM</b>	Segment Access Manager	
<b>SAM</b>	Self Assembling Material	
<b>SAM</b>	Self Automated Machine	
<b>SAM</b>	Self-Assembled Monolayer	
<b>SAM</b>	Semi-Analytic Model (galactic modeling)	
<b>SAM</b>	Sensor Arrays and Multichannel Signal Processing	
<b>SAM</b>	Serbia and Montenegro	
<b>SAM</b>	Serial Access Memory	
<b>SAM</b>	Service Access Multiplexer (Bellcore)	
<b>SAM</b>	Service Account Manager	
<b>SAM</b>	Service Adaptation Module (Tachion)	
<b>SAM</b>	Service Assurance Manager (Smarts)	
<b>SAM</b>	Service Automation Module (Opsware)	
<b>SAM</b>	Servicing And Maintenance	
<b>SAM</b>	Serving Area Multiplex Sites	







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## Image FlashPlate Streptavidin coated, 40 plates

### Product categories:

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### Unit Size

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RMP110

Image FlashPlate Streptavidin coated, 40 plates

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### Product Information

#### Description:

Basic Image FlashPlate pre-coated with Streptavidin and a blocking protein. For capture of biotinylated molecules. Widely used for many types of enzyme assays.

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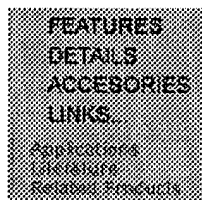
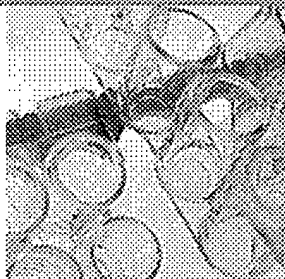
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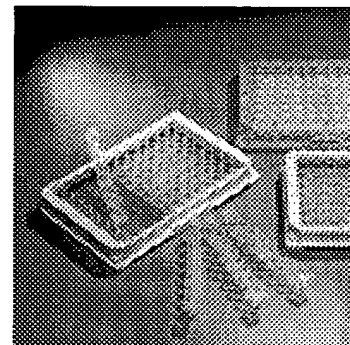




## Immunology Streptavidin Coated Plates

### Streptavidin Coated Plates

- Ideal for binding of biotinylated biomolecules such as peptides, antibodies, oligonucleotides or haptens
- Streptavidin coated area of 154 mm<sup>2</sup> (area covered by a volume of 200 µl)
- Binding capacity for biotin of at least 13 pmol/well\*
- Stable at room temperature
- General coating protocol available on request
- Available in C96 Plates or C8 well strips
- Other formats available on request



\* Depending on size or sterical properties of a given biomolecule the actual binding capacity might be different.

### Details

#### Streptavidin Coated Plates

Polystyrene, 96 wells

External dimensions 128 x 86 mm

Cat. No.	236001	236004
Configuration	C96	C8
Colour	Transparent	Transparent
Total volume, µl/well	350	350
Coated well volume, µl	200	200
Units per pack/case	1/15	1/15
Availability	Not American	Not American

### Accessories

#### Accessories for Streptavidin Coated Plates

Cat. No.	430805	430082
Description	8 Well strip cap	8 Well strip cap
Material	Polyethylene	Polyethylene
Sterile	+	-
Units per pack/case	12/120	12/120
Availability	Not American	Not American

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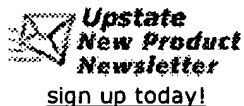
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**Tested Applications:** enzyme assay

**Application Notes:**  
For use in plate assays.



For general laboratory.  
FOR *IN VITRO* USE ONLY.

# StreptaWell\*

## *Instruction Manual*

Version 1, January 2003

Store the plates at 2–8°C

	StreptaWell*	StreptaWell High Bind*
Plate Type	Cat. No. (pack size)	Cat. No. (pack size)
96-wells, transparent, C-bottom	1 734 776 (15 Plates)	1 989 685 (15 Plates)
12 × 8-well strips and frame, transparent, C-bottom	1 664 778 (5 Plates)	1 645 692 (5 Plates)
12 × 8-well strips and frame, transparent, nuclease-free, C-bottom	1 768 000 (5 Plates)	
384-wells, transparent, C-bottom	1 989 669 (5 Plates)	
96-wells, white, C-bottom	1 989 707 (15 Plates)	1 989 693 (15 Plates)
12 × 8-well strips and frame, white, C-bottom	1 602 861 (5 Plates)	1 989 715 (5 Plates)
384-wells, white, C-bottom	1 989 677 (5 Plates)	
96-wells, black, C-bottom	1 734 784 (15 Plates)	
12 × 8-well strips and frame, black, C-bottom	1 602 837 (5 Plates)	

\*covered by EP Patent EP-B 0269092 and US Patent US 5,061,640  
granted to Roche Applied Science



## 1. Preface

<b>1.</b>	<b>Preface</b>	<b>2</b>
1.1	Table of contents	2
<b>2.</b>	<b>Introduction</b>	<b>3</b>
2.1	Product overview	3
2.2	Product characteristics	5
<b>3.</b>	<b>Procedures and required materials</b>	<b>7</b>
3.1	Before you begin	7
3.2	Labeling of molecules	8
3.3	ELISA Protocol	9
3.4	Optimizing ELISA protocols	10
<b>4.</b>	<b>Appendix</b>	<b>11</b>
4.1	Trouble-shooting	11
4.2	Related products	12
4.3	Coating service	14

## 2. Introduction

### 2.1 Product overview

#### StreptaWell

Streptavidin-coated microplates are available in two binding capacities:

- **StreptaWell** (regular binding capacity) and
- **StreptaWell, High Bind** (high binding capacity).

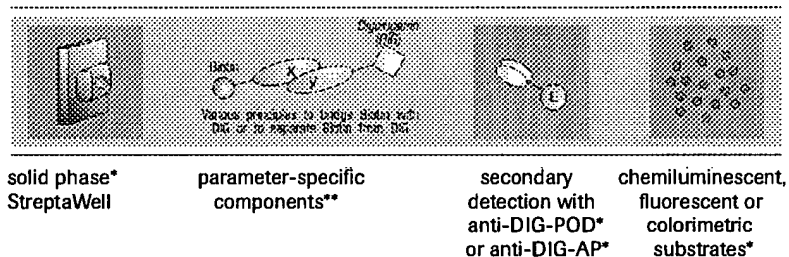
The different binding capacities result from different coating procedures using different starting materials.

#### Streptavidin-based assay system

The modular streptavidin-based assay system is composed of more than 100 different items which enable the set up of almost any biochemical assay. The system consists of universal modules and parameter-specific components (Fig.1). The universal modules are identical for most applications, comprised of a streptavidin-coated microplate (StreptaWell), a Roche Applied Science proprietary tracer system anti-digoxigenin-enzyme conjugate, and a set of different substrate alternatives to generate a chemiluminescent, fluorescent or colorimetric signal.

The parameter-specific components (which have to be designed according to the parameter of interest) are biotin-labeled for immobilization purposes, and digoxigenin (DIG)-labeled for detection and quantification. The techniques associated with labeling the parameter-specific components like peptides, proteins, and nucleic acids are well established.

Protocols, highly convenient kits, and reagents are all available from Roche Applied Science.



\* Universal Modules, available in various DIG Detection ELISAs

\*\* Parameter-specific components, can be labeled via Nucleic Acids- and Protein-Labeling Kits, available from Roche Applied Science

**Fig. 1: Principle of the Modular Streptavidin-based Screening System**

## 2.1 Product overview, Continued

### Detection principles

StreptaWell can be used with the following different detection principles:

StreptaWell	Detection		
	Colorimetric	Chemiluminescent	Fluorescent
transparent	++	-	+
white	-	++	++
black	-	+	+

### Application

StreptaWell can be used to study:

- Enzyme activities
- Immunoassays
- Protein modifications
- Protein-Protein interactions / Receptor Binding
- Protein-Nucleic acid binding
- Nucleic acid amplification and hybridization
- Cell products (cytokines, steroids etc.)
- Reporter genes

## 2.2 Product characteristics

### Specifications

The following table gives an overview about the coating specifications.

	<b>StreptaWell 96</b>	<b>StreptaWell 96 High Bind</b>	<b>StreptaWell 384</b>
SA-coated area (indicated as volume)	≥ 300 µl	≥ 300 µl	≥ 60 µl
Blocked volume	≥ 320 µl	≥ 320 µl	≥ 75 µl
Total biotin binding capacity* (competition assay)	≥ 5 ng/well ≥ 20 pmol/well ≥ 70 nM	≥ 20 ng/well ≥ 80 pmol/well ≥ 200 nM	≥ 1 ng/60 µl well ≥ 4 pmol/60 µl well ≥ 70 nM
Total binding capacity for biotin-labeled antibodies	1.5 µg/well	1.5 µg/well	0.3 µg/60 µl well
Coating variance between individual wells	< 5%	< 5%	< 8%
CV between different plates	< 10%	< 10%	< 15%
SA-leaching	< 5 ng/well	< 5 ng/well	< 1 ng/60 µl well

\* well: 300 µl

*continued on next page*



## 2.2 Product characteristics, Continued

<b>Homogeneity and production variances</b>	<p>The proprietary coating process guarantees homogenous and reproducible coatings with unique features:</p> <ul style="list-style-type: none"> <li>• little variances between individual wells (intra-assay-variance)</li> <li>• little variances between different plates (inter-assay-variance)</li> <li>• high lot-to-lot reproducibility</li> <li>• high signal-to-noise ratio</li> <li>• low background</li> <li>• almost no leaching</li> </ul>
<b>Storage/stability</b>	<p>The unopened plates are stable at 2-8°C through the expiration date printed on the label.</p> <p><b>Note:</b> Store dry and protected from light.</p>
<b>Binding capacity</b>	<p>The biotin binding capacity as given under specifications is identical to the number of biotin binding sites present. Depending on the size and sterical properties of a given biomolecule, the actual molar binding capacity may often be below this maximum value.</p>
<b>Factors influencing binding capacity</b>	<p>Even though the binding capacity could be reduced to some extent, the integrity of the coating and the stability of the streptavidin-biotin interaction has proven to be remarkably resistant to a variety of harsh conditions:</p> <ul style="list-style-type: none"> <li>• buffers generally used in molecular biology like SSC, TEN, RIPA, TBS, etc.</li> <li>• 4 M Guanidinium-thiocyanat, 1 h, 15-25°C</li> <li>• 4 M Urea, 37°C, 1 h</li> <li>• 50% formamide, 56°C, 1 h</li> <li>• 1% SDS, 37°C, 1 h. (Some detergents may influence the properties of StreptaWell in concentrations above 0.1 to 1%.)</li> <li>• pH 4-10,</li> <li>• elevated temperatures up to 75°C. (Do not heat above 75°C.)</li> </ul>
<b>Sensitivity</b>	<p>Potential sensitivity-limiting factors for a microplate assay are:</p> <ul style="list-style-type: none"> <li>• affinity of the specifically interacting components</li> <li>• sensitivity of the detection system</li> <li>• non-specific binding (signal-to-noise).</li> </ul> <p>Therefore maximum sensitivity of the detection method can only be achieved if the affinities of the interacting components are not limiting for the assay and the total of non-specific signal is well below 0.1 % of total signal.</p>
<b>Pigmentation</b>	<p>The pigmentation of the microplates is to prevent 'cross-talk' (light exchange) between individual wells. Both, the white and the black plates have cross-talk-levels well below 0.1%. Due to light-reflection the signal intensity in white plates is about 10 times higher as obtained from black plates. If the background-signal is low, this could lead to an increase in sensitivity.</p>
<b>Caution</b>	<p>Do not sonify microplates or wells.</p>

### 3. Procedures and required materials

#### 3.1 Before you begin

##### General

StreptaWell plates are ready to use as supplied. No extra rehydration step is needed.

##### Additional substrates required

In the following table you will find substrates available from Roche Applied Science suitable for the different detection principles:

StreptaWell	Substrates		
	Colorimetric	Chemiluminescent	Fluorescent
transparent	4-Nitrophenyl-phosphate CPRG ABTS BM Blue POD TMB	-	+
white	-	BM CL ELISA Substrates: (AP) (POD)	AttoPhos
black	-	+	+

### 3.2 Labeling of molecules

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**Additional reagents required**

For labeling of biomolecules or biomolecule complexes, highly convenient labeling reagents or ready-to-use labeling kits are available, see Related Products.

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**Labeling of proteins, peptides and small molecules**

Labeling of proteins, peptides and small molecules like haptens can be performed under mild conditions via free amino groups, sulfhydryl groups, disulfide bridges or oxidized sugar residues (aldehyde or keto groups). In general it is recommended to label proteins via free amino groups (lysyl residues). Labeling with biotin normally has no effect on the properties of proteins. In small molecules like haptens and oligopeptides, the label has to be conjugated to a part of the molecule, which is not essential for function. Often a spacer structure between label and hapten (or ligand) is advantageous to allow optimal interaction with antibodies (or receptors).

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**Labeling of nucleic acids**

Nucleic acid labeling may be performed chemically or enzymatically with reagents or kits. In molecular biology, double labeling with biotin and digoxigenin is often used to analyze nucleic acids in polymerisation and hybridization experiments.

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### 3.3 ELISA Protocol

- Before you begin**
- When possible, allow biotinylated components to bind to streptavidin under physiological buffer conditions. For more stringent conditions see section 3.4.
  - Immobilization of biomolecules via streptavidin/biotin interaction is at least as effective as direct coating to physically activated surfaces. In many cases, e.g. small molecules, oligonucleotides or peptides, the binding of biotinylated components will be much more efficient.
  - After washing out excess biotinylated substance, all further steps may follow the standard protocol as optimized for a particular parameter. Volumes and general conditions are given below.

**General ELISA protocol**

In the following table please find the components needed for a general ELISA procedure:

Step	Buffers	Volume	Time/Temp.(°C)
Binding of biotinylated component	PBS or TBS containing 0.1% BSA	50-100 µl	15-60 min/15-25°C or 35°C
Washing steps	PBS or TBS containing 0.1% BSA and/or further additives	300 µl (each wash cycle)	3-5 × with 5 min incubation between individual washes/ 15-25°C
Antibodies, antigen, etc. incubations	PBS or TBS containing 0.1% BSA and/or further additives, depending on the components used	100-150 µl	60 min/ 15-25°C or 35°C
Secondary detection component		200 µl	
Colorimetric-, chemiluminescence-or fluorescence substrate solutions	Prepare solutions according to the manufacturers protocol or use ready-to-use reagents	250 µl (including volume of trigger solutions, if required)	Depending on enzyme/ substrate system/ 15-25°C

### 3.4 Optimizing ELISA protocols

#### Antibody-conjugate concentration

When changing to fluorescent or chemiluminescent detection, the concentrations of the conjugates often have to be adapted. However, at the first attempt, the conjugate concentration should be used as recommended by the supplier or as optimized for a particular colorimetric assay. If the conjugate contributes to non-specific binding, its concentration may be lowered down to 1:10.

#### Reduction of non-specific binding

Optimizing non-specific binding might be a prerequisite for highly sensitive detection. To reduce background, either additional components may be added to washing-, incubation- and conjugate buffers and/or the concentrations of the specific interacting components may be lowered.

The following additives may be used:	Additives / Concentration
Salt	0.5-1.0 M NaCl
Complexing agent	1-5 mM EDTA
Detergent	0.05-0.1% Tween <sup>®</sup> 20
Protein	0.1-1% BSA, serum, casein, milk powder

#### Washing conditions

Most interactions which contribute to non-specific binding are of low/ intermediate affinity and therefore reversible in character. Prolonged intervals between individual washes (we recommend at least 3 repeated washes) favor dissociation from non-specific binding sites.

#### Handling very concentrated samples

Samples exceeding the measuring range should be diluted with incubation buffer and the ELISA should be repeated. This dilution factor has to be taken into account when calculating the content.

## 4. Appendix

### 4.1 Trouble-shooting

Problem	Possible cause	Recommendation
Weak or no signal		Check instrument settings.
		Check activity of marker enzyme/ molecule.
	Water ingredients that influence the test negatively.	Always use double distilled water for reconstitution and preparing the working solutions; take care that the water is not microbially contaminated!
	Interference of buffer components with substrate	Check conjugate buffer for incompatible components (e.g. NaN <sub>3</sub> , SH-reagents).
	Inadequate incubation time and temperature	Check protocol (incubation times/ temperatures, buffer conditions, etc.) and concentrations of primary antibody or antigen.
	Substrate or vial used to aliquot substrate contaminated	Check substrate reagent for storage conditions and biological contamination. Use freshly prepared reagent. Check integrity of positive control.
High background signal	Washing procedure not efficient	Prolong washing procedure (number of washes, interval between washes).
	Non-specific interaction of buffer additives	Try different additives with the washing/ incubation buffers to block non-specific interactions.
	Inadequate concentration of detection component, e.g. antibodies	Modulate concentrations for detection components, e.g. primary/secondary antibody.

## 4.2 Related products

Product	Pack Size	Cat. No.
<b>Streptavidin (SA)-coated and Anti-DIG-coated Tubes and Magnetic Particles</b>		
SA-coated Tubes	4 × 25 tubes	1 602 845
SA-coated PCR Tubes (Strips)	24 strips of 8 × 0.2 ml tubes and caps	1 741 772
SA Magnetic Particles	2 ml 10 ml	1 641 778 1 641 786
Anti-Digoxigenin Magnetic Particles	2 ml	1 641 751
Magnetic Particles Separator	1 separator (for 4 × 1.5 ml tubes). 1 separator (for 3 × 15 ml or 50 ml tubes; or for a 96 well MP).	1 641 794 1 858 025
<b>Colorimetric Substrates</b>		
CPRG	250 mg	884 308
ABTS	2 g	102 946
ABTS Solution	3 × 100 ml	1 684 302
ABTS Tablets	20 tablets (5 mg for 5 ml)	1 204 521
ABTS Tablets	20 tablets (50 mg for 50 ml)	1 112 422
BM Blue POD Substrate, precipitating	100 ml	1 442 066
BM Blue POD Substrate, soluble	100 ml	1 484 281
TMB	1 g	784 974
4-Nitrophenylphosphate	5 g	107 905
4-Nitrophenylphosphate	10 tablets	726 923
<b>Chemiluminescent Substrates-BM CL ELISA Substrates</b>		
BM Chemiluminescence ELISA Substrate AP	150 ml	1 759 779
BM Chemiluminescence ELISA Substrate POD	250 ml	1 582 950
<b>Fluorescent Substrates</b>		
AttoPhos	for 1800 wells	1 681 982

*continued on next page*

#### 4.2 Related products, Continued

Product	Pack Size	Cat. No.
<b>Labeling proteins</b>		
Biotin Protein Labeling Kit	1 kit	1 418 165
DIG Protein Labeling Kit	1 kit	1 367 200
<b>Labeling nucleic acids</b>		
Biotin Chem Link	1 set	1 812 149
Biotin High Prime	100 µl	1 585 649
Biotin RNA Labeling Mix	40 µl	1 685 597
DIG Chem-Link Labeling and Detection Set	1 set	1 836 463
DIG DNA Labeling Kit	1 kit	1 175 033
DIG Oligonucleotide 5'-End Labeling Set	1 set	1 480 863
DIG Oligonucleotide 3'-End Labeling Kit	1 kit	1 362 372
DIG High Prime	160 µl	1 585 606
PCR DIG Labeling Mix <sup>Plus</sup>	2 × 250 µl	1 835 289
PCR ELISA DIG Labeling <sup>Plus</sup>	2 × 250 µl	1 835 297
PCR DIG Probe Synthesis Kit	1 kit	1 636 090
DIG RNA Labeling Kit	1 kit	1 175 025

#### Additional related and required products

For Secondary detection- or Anti-species antibodies and Anti-DIG- or Anti-Fluorescein-conjugates please see **Roche Applied Science Biochemicals Catalog**.



### 4.3 Coating service

#### What the coating service offers

Customized Microplate Coating is a new service established to assist our customers. With this new service, customers can benefit from Roche's outstanding expertise in immunochemistry. Our proprietary coating technologies can be applied to diverse biomolecules of your choice:

- **Coating process according to GMP and ISO 9001**
- **Lot sizes of 200 (minimum) to 2000 plates**
- **Very high production capacities**
- **Delivery time  $\leq$  8 weeks**
- **Broad range of solid support possible:**  
96-well and 384-well microplates (transparent, white, black)  
PCR plastic ware, plates or tubes
- **Common Coatings Include:**  
Streptavidin, Anti-CAT, Anti-DIG, Anti-Fluorescein, Anti- $\beta$ -Gal, Anti-GST, Anti-HA (3 F 10), Anti-hGH, Anti-His6, Anti-Human IgG, Anti-Human-IgM, Anti-Mouse Ig, Anti-Rabbit IgG
- **Coating Procedure:**  
Various procedures available, depending on intended use: Nucleic acid or Protein screens. Samples will be provided.

Requirements and conditions for Customized coatings (e.g. antibodies, antigens or haptens) are available on request from our Coating Service department. Please contact our specialized local representative.

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United States

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United States

Roche Molecular Biochemicals is your premier partner in PCR, nucleic acid isolation, apoptosis, tissue dissociation, protease inhibition, chemiluminescent and fluorescent detection. We offer systems like LightCycler, MagNA Pure LC, and Lumilimager.

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